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p53 Binding Areas

The present invention relates to p53 binding areas (regions) on a CD95 receptor DNA and to the use of the p53 binding regions for influencing apoptosis and/or for identifying substances suitable for this purpose.

p53 is a tumor suppressor which is induced in the case of DNA damage. It then activates target genes so as to achieve growth stand-still in the cells having DNA damage followed by the repair of the DNA damage or death of the cells. The latter is due to apoptosis.

A chemotherapy is to cause DNA damage in tumor cells. This damage shall then lead to the induction of p53 and ultimately to the death of the tumor cells. However, it shows frequently that certain tumor cells are resistant to chemotherapeutic agents or become resistant thereto after a short treatment duration. The reason why this is the case is not really known thus far.

Therefore, it is the object of the present invention to provide a product by which the resistance to chemotherapeutic agents can be investigated and optionally influenced.

According to the invention this is achieved by the subject matters defined in the claims.

The present invention is based on applicant's insights that the induction of p53 by chemotherapeutic agents directly

activates apoptosis. In particular, applicant found that p53 activates CD95-mediated apoptosis in that p53 induces both the expression of the CD95 ligand and that of the CD95 receptor. Applicant also found that p53 binds to CD95 receptor DNA via p53 binding regions. He also identified such bindings regions in intron 1 and/or the promoter of the CD95 receptor DNA. Moreover, applicant recognized that resistance to chemotherapeutic agents may be due to the fact that p53 can no longer bind to the above p53 binding regions (cf. Table 1 and figures 1-6).

According to the invention applicant's insight are used to provide a p53 binding region of a CD95 receptor DNA.

The term "p53 binding region" comprises any region of a CD95 receptor DNA to which a p53 may bind and activate the CD95 receptor DNA, i.e. may induce it to transcribe. The term "p53" comprises p53 in wild-type form as well as p53 in modified form which still has the above function. A p53 binding region according to the invention may be identified and provided by common methods. It is favorable to cleave a CD95 receptor DNA (cf. Behrmann, I. et al., Eur. J. Immunol. 24 (1994), 3057-3962) by Sau 3A1 and insert the fragments in the BamHI site of pBlueScript II KS^{+}. The cloned CD95 receptor DNA fragments are inserted in DNA binding experiments which use cell extracts from the tumor cells, e.g. H1299, Hep3B, HepG2 or Huh7, which had been transfected beforehand with a p53-coding expression vector, e.g. pCMVp53wt. Bound DNA fragments are fused with a reporter DNA, e.g. luciferase DNA. This may be made e.g. in the expression vectors pGL3-Basic (Promega company) or pTATA-LUC (Wirth, T., Würzburg, Germany). Resulting expression plasmids are tested in luciferase activity tests for their capacity of being activable.

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In a preferred embodiment, a p53 binding region comprises the sequence of figure 4 (p53 Be sequence) and/or figure 5 (one or more of the p53 Be sequences) or a sequence differing therefrom by one or more base pairs. The expression "a sequence differing by one or more base pairs" comprises any sequence of a CD95 receptor DNA which hybridizes with the DNA of figure 4 and/or figure 5 and to which a p53 may bind and which may activate the CD95 receptor DNA. The sequence may differ from the DNA of figure 4 and/or figure 5 by additions, deletions, substitutions and/or inversions of one or more base pairs. The expression "hybridization" refers to hybridization under common conditions, in particular at 20°C below the melting point of the sequence.

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In a particularly preferred embodiment a p53 binding region comprises the sequence of figures 7, 8, 9, 10, 11, 12, or 13, the sequences of figures 11, 12 and 13 being variations of the sequences of figures 8, 9, and 10, respectively. Furthermore, the sequences of figures 7, 8, 9 and 10 are explained in figure 14.

A p53 binding region according to the invention may be present as such or in combination with any other DNA. For example, a p53 binding region according to the invention may be present in a vector, optionally in combination with a reporter DNA, e.g. luciferase DNA. Preferred combinations are the DNA constructs CD95(Ps)-LUC, CD95(P)-LUC, CD95(I+SV)-LUC, CD95(Ps+I)-LUC, p1139, p1140, p1141, p1142, p1140 IMI, p1140 IMII, p1140 IMIII, p1140 IMIV, p1141 IMIII, p1141 1p53, p1141 2p53, p1141 3p53, p1141 ΔBgl, p1141 ΔSpe, p1141 ΔMph, p1142 TAG, p1142 IMIII, p1142 ΔBgl, p1142 ΔSpe and p1142 ΔMph, in which a p53 binding region according to

the invention is present in the expression vectors pGL3-Basic and/or pTATA-LUC. As to the DNA constructs CD95(Ps)-LUC, CD95(P)-LUC, CD95(I+SV)-LUC, CD95(Ps+I)-LUC, reference is made to Example 3 and figure 6. The DNA constructs p1139, p1140, p1141, p1142, p1140 IMI, p1140 IMII, p1140 IMIII, p1140 IMIV, p1141 IMIII, p1141 1p53, p1141 2p53, p1141 3p53, p1141 ΔBgl, p1141 ΔSpe, p1141 ΔMph, p1142 TAG, p1142 IMII, p1142 ΔBgl, p1142 ΔSpe, and p1142 ΔMph contain the sequences indicated in figures 7, 8, 9 or 10, i.e. p53 binding regions or variations thereof (cf. figures 11, 12, and 13). The DNA constructs p1139, p1140, p1141 and p1142 are preferred and were deposited with DSMZ (*Deutsche Sammlung für Mikroorganismen und Zellen* [German-type collection of microorganisms and cells]) on September 24, 1999, i.e. p1139 under DSM 13075, p1140 under DSM 13062, p1141 under DSM 13063 and p1142 under DSM 13064.

A further subject matter of the present invention is a kit comprising a p53 binding region according to the invention (a) and common auxiliary ingredients (b), such as buffers, solvents, carriers, controls, etc. One or more representatives of the p53 binding region may be present. The above explanations also apply correspondingly.

The present invention enables mechanisms resulting when DNA is damaged to be investigated on a molecular level. Such mechanisms comprise the response of the cells to eliminate the DNA damage or to kill themselves. The latter is an apoptotic process. The present invention enables mechanisms resulting in a chemotherapy to be investigated. In particular, it is possible to investigate the cause of resistances to chemotherapeutic agents. For example, it can be determined by means of a p53 binding region according to

the invention whether tumor cell-derived p53 is still capable of inducing apoptosis.

The present invention is also suitable to identify and provide substances capable of influencing apoptosis. This influence may be an induction or an inhibition. For this purpose, it is favorable to introduce into cells a p53 binding region according to the invention in combination with a reporter DNA, add thereto the substances to be identified and select them for the transcription-activating or transcription-inhibiting effect of the substances. p53 binding regions may be activated or inhibited in a CD95 receptor DNA by means of these substances and therefore induce or inhibit apoptosis.

Thus, the present invention provides products or means serving for influencing apoptotic processes. This is of great significance, since apoptotic processes are modified in many diseases. For example, the apoptosis rate of viral, liver and neurodegenerative diseases is increased whereas it is lowered in autoimmune and tumoral diseases. Thus, the present invention is the possibility of therapeutically influencing these diseases. An application in a diagnostic respect is also useful, in particular if a p53 gene therapy is carried out in connection with the above-mentioned diseases and the vectors used for this purpose are tested for effectiveness, availability, etc., by means of the vectors according to the invention.

Brief description of the drawings

Figure 1 shows the expression of the CD95 receptor in tumor cells after treating them with chemotherapeutic agents. Clinically relevant concentrations of the

chemotherapeutic agents are marked with an asterisk. The tumor cells express p53, no p53 (-/- p53) or p53 disturbed as regards the binding to an inventive p53 binding region of a CD95 receptor DNA (mt p53).

Figure 2 shows the response of tumor cells treated with chemotherapeutic agents to the induction of apoptosis by CD95 receptor stimulation.

Figure 3 shows the expression of the CD95 receptor in tumor cells treated with a chemotherapeutic agent, the tumor cells expressing p53 only after transfection with an expression plasmid coding for p53.

Figure 4 shows a p53 binding region according to the invention (p53 BE) within intron 1 of a CD95 receptor DNA.

Figure 5 shows a p53 binding region according to the invention (p53 BE) within the promoter of a CD95 receptor DNA comprising 9 exons. The promoter has three p53 binding regions.

Figure 6 shows the expression of a luciferase DNA after the binding of p53 to a p53 binding region according to the invention within an expression plasmid containing the luciferase DNA.

Figure 7 shows the sequence of a p53 binding region according to the invention, the sequence comprising the nucleotides 1-720 of intron I of the CD95 receptor DNA. The p53-BE sequence is marked in boldface.

Figure 8 shows the sequence of a p53 binding region according to the invention, the sequence comprising nucleotides 448 - 2154 of the promoter, exon I and the nucleotides 2223 - 2827 (correspond to nucleotides 116 - 720 of the sequence of figure 7) of intron I of the CD95 receptor DNA. The p53-BE sequences are marked in boldface.

Figure 9 shows the sequence of a p53 binding region according to the invention, the sequence comprising nucleotides 1 - 2154 of the promoter, exon I and nucleotides 2223 - 2827 of intron I of the CD95 receptor DNA. The p53-BE sequences are marked in boldface.

Figure 10 shows the sequence of a p53 binding region according to the invention, the sequence comprising nucleotides 1 - 2154 of the promoter, exon I together with its 3' region and nucleotides 2223 - 2820 of intron I together with its 5'-region of the CD95 receptor DNA. The p53-BE sequences are marked in boldface.

Figure 11 shows variations in the p53 binding region of figure 8, the variations being point mutations in intron I of the CD95 receptor DNA.

Figure 12 shows variations in the p53 binding region of figure 9, the variations being point mutations in intron I and in the promoter as well as deletions in the promoter of the CD95 receptor DNA.

Figure 13 shows variations in the p53 binding region of figure 10, the variations being point mutations in intron I and in exon I as well as deletions in the promoter of the CD95 receptor DNA.

Figure 14 shows a physical map of p53 binding regions according to the invention, (a) being the binding region of figure 7, (b) being that of figure 8, (c) being that of figure 9, and (d) being that of figure 10.

The present invention is explained by the below examples.

Example 1: Detection of the expression of the CD95 receptor in tumor cells treated with chemotherapeutic agents (A) and of the response of these tumor cells to the induction of apoptosis by CD95 receptor stimulation (B).

(A) The tumor cells HepG2 (human hepatoblastoma), AGS (colon carcinoma) HS746T (gastric carcinoma), MCF-7 (breast cancer), Hep3B (human hepatoblastoma), Huh7 (hepatocellular carcinoma), and HT29 (colon carcinoma) are treated with the chemotherapeutic agents bleomycin, 5-fluorouracil, methotrexate, mitomycin and cisplatin. HepG2, AGS, HS746T and MC-7 express a p53 which binds to a p53 binding region according to the invention. Hep3B expresses no p53. Huh7 and HT29 express a p53 which is disturbed as regards its binding to a p53 binding region according to the invention. The expression of the CD95 receptor is determined by FACScan. To this end, a biotinylated anti-APO-1 (CD95 receptor) antibody and quantum red-streptavidine (Sigma

company) are used as a second reagent for an indirect immunofluorescence (cf. figure 1).

It shows that only the tumor cells HepG2, AGS, HS746T and MCF-7 whose p53 binds to a p53 binding region according to the invention, have CD95 receptor expression.

(B) The tumor cells HepG2, Huh7 and Hep3B (cf. (A)) are treated with the chemotherapeutic agents 5-fluorouracil, methotrexate, mitomycin, cisplatin, mitoxantrone, doxorubicin, etoposide and cyclophosphamide for 48 h or another 24 h in combination with 100 ng/ml IgG3 anti-APO-1 antibodies. The antibody effects CD95 receptor stimulation. The living cell fraction is determined. For this purpose, the MTT test is carried out determining the ability of living cells to reduce soluble yellow tetrazolium salt (MTT) to form blue formazan crystals (cf. figure 2).

It shows that only the tumor cell HepG2 whose p53 binds to a p53 binding region according to the invention responds more intensely to apoptosis induction.

Example 2: Detection of the expression of the CD95 receptor in bleomycin-treated tumor cells, the tumor cells expressing p53 only following transfection.

The tumor cells Hep3B (0.6×10^6 cells) which usually express no p53, are transfected with 1 µg of the expression vector pCMVp53wt coding for p53 by means of the calcium phosphate coprecipitation method. Thereafter, the tumor cells are treated with bleomycin.

The expression of the CD95 receptor is determined by FACScan (cf. Example 1(A); figure 3).

It shows that an expression of the CD53 receptor is obtained by the expression of p53.

Example 3: Detection of the expression of luciferase DNA by p53 binding to a p53 binding region according to the invention.

Expression plasmids are produced, the expression vector pGL3-Basic being used as the vector. The following CD95 receptor DNA/luciferase-DNA constructs are inserted in this vector:

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CD95(Ps)-LUC

The luciferase-DNA is linked via its 5' end with a 1.43 kb promoter region and the 5' end of exon 1 of the CD95 receptor DNA (HindIII-SacII fragment, cf. figures 5 and 6).

Sub B4

CD95(P)-LUC

The luciferase DNA is linked via its 5' end with a 1.9 kb promoter region and the 5' end of exon 1 of the CD95 receptor DNA (cf. figures 5 and 6).

Sub B5

CD95(I+SV)-LUC

The luciferase DNA is linked via its 5' end with the "minimum" SV40 promoter and a 0.7 kb intron 1 fragment of the CD95 receptor DNA (cf. figures 4 and 6).

CD95(Ps+I)-LUC

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The luciferase DNA is linked via its 5' end with a 0.7 kb intron 1 fragment and a 1.43 kb promoter region of the CD95 receptor DNA (cf. figures 4 and 6).

The above expression plasmids (1 µg each) are transfected in Hep3B tumor cells. The expression vector pCMVp53wt (100 ng each) is also transfected. Both transfections are effected by the calcium phosphate coprecipitation method. A common luciferase test is carried out (cf. figure 6).

It shows that the DNA constructs CD95(PS)-LUC and CD95(P)-LUC serve for achieving an activation of luciferase which is about 2 times to that of a control. An even more intense activation is obtained when the DNA construct CD95(I+SV)-LUC and in particular the DNA construct CD95(PS+I)-LUC are used. In the latter case, the activation has a factor of about 50.

Table 1

Induction of p63, the CD95 receptor and of apoptosis by chemotherapeutic agent

Chemotherapeutic agent	Mode of action		P53 induction	Induction of apoptosis	CD95 receptor induction	Increased response to induction of apoptosis by CD95 receptor stimulation
Fluorouracil	Antimetabolite	Pyrimidine antagonist	+	+	+	+*
Methotrexate	Antimetabolite	Folic acid antagonist	+	+	+	+*
Mitomycin	Alkylation		+	+	+	+*
Cisplatin	Alkylation		+	+	+	+*
Cyclophosphamide	Alkylation		+	+	+	+*
Mitoxantron	Intercalation		+	+	+	+*
Doxorubicin	Intercalation		+	+	+	+*
Etoposide	Mitotic blocking	Inhibition of topoisomerase II	+	+	+	+*
Bleomycin	Inhibition of DNA polymerase		+	+	+	+*

* test for synergism between CD95 receptor stimulation by anti-APO-1 and simultaneous chemotherapeutic treatment: $p < 0.0001$